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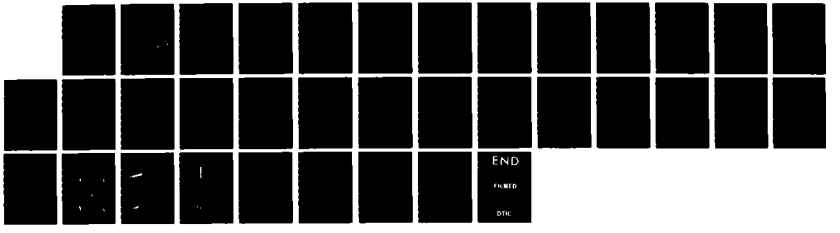

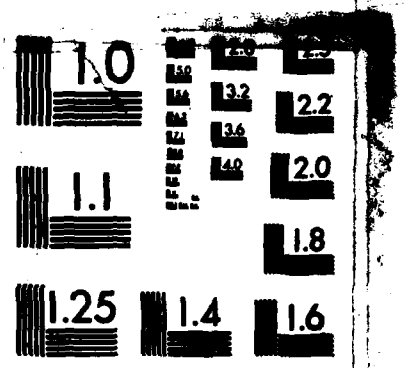

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Spectral "Fingerprinting" of Phytoplankton Populations by Two-Dimensional Fluorescence and Fourier-Transform-Based Pattern Recognition

by

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SPECTRAL "FINGERPRINTING" of PHYTOPLANKTON POPULATIONS by TWO-DIMENSIONAL FLUORESCENCE and FOURIER-TRANSFORM-BASED PATTERN RECOGNITION

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Abstract

The selectivity of fluorescence spectroscopy is exploited for the characterization of marine algae. Two-dimensional, digital images of in vivo fluorescence intensity versus excitation and emission wavelengths, called excitation-emission matrices (EEMs), are used as spectral "fingerprints" for marine phytoplankton populations. Fourier-transform-based pattern recognition is described along with its inherent strengths and weaknesses for the analysis of natural populations. The EEMs of unknown algae are compared to a library of standard EEMs representing 23 algal species and 6 classes with better than 80% accuracy. The EEMs acquired under different physiological conditions are used in determining pattern recognition reliability. The potential for fingerprinting mixed populations and oceanographic regions is also discussed.

Introduction

A considerable amount of work has been conducted through the years in documenting the pigment compositions of phytoplankton and their taxonomic relationships (Rowan, 1981; Parsons et al., 1984). Most of the algal divisions are unique in their specific combination of pigments. These pigments can be divided into three main classes that will be referred to in this manuscript as the chlorophylls, carotenoids, and phycobilins. Chlorophyll a provides the universal link between these three groups since it is the only pigment present in all algal species and because of its unique role in photosynthesis. Acting as an energy storehouse, it accepts excitation energy from the so-called accessory pigments (chlorophylls b and c, carotenoids, and phycobilins) and channels it into the photosynthetic process. This allows photosynthesis to continue over a broad range of ambient light wavelengths.

The determination of individual pigment concentrations and especially that of chlorophyll a has provided useful information concerning the amount and stage of development of phytoplankton populations (Ryther, 1956; Ryther and Yentsch, 1957; Moreth and Yentsch, 1970). Spectroscopic characterization of phytoplankton populations by cellular pigment composition is well documented (Richards and Thompson, 1952; Parsons and Strickland, 1963; Yentsch and Menzel, 1963; Parsons, 1961). Richards and Thompson (1952) developed a spectrophotometric method for the quantitation of the chlorophylls after extraction into 90% acetone. This method is often called the trichromatic method since it requires the measurement of absorbance at three wavelengths. Yentsch and Menzel (1963) later

introduced a fluorometric method that was simpler and more sensitive than the previous technique. Later studies used continuous in vivo monitoring of chlorophyll a by fluorescence (Lorenzen, 1966) that was capable of providing additional information concerning the organism (Slovacek and Hannan, 1977). These in vivo monitoring techniques capitalize on the low detection limits of fluorescence. However, fluorescence spectroscopy offers more than low detection limits. Fluorescence is considered a multiparametric technique since there are two spectra (excitation and emission) that define the total fluorescence spectrum. This total fluorescence spectrum is often called an excitation-emission matrix (EEM) (Warner et al., 1976; Johnson et al., 1977). Since there are two dimensions of information in an EEM, very subtle differences between fluorescent samples can be distinguished. Therefore, just as a photograph can be used for identification, an EEM can be used to identify or "fingerprint" complex fluorescent samples. Specific examples of the fingerprinting capabilities possible with EEMs include the examination of mixtures of polynuclear aromatic hydrocarbons (Fogarty and Warner, 1980), the identification of bacteria (Shelly et al., 1980 a,b), and the differentiation of normal and cancerous mammalian cells (Rossi et al., 1982). Several data handling methods have been developed to help interpret EEMs but these have primarily focused on the deconvolution and quantitative analysis of multicomponent EEMs (Fogarty and Warner, 1981; Warner et al., 1977; Ho et al., 1978; Ho et al., 1980). A number of quantitative methods for the determination of planktonic pigments by fluorescence detection also have been published (White et al., 1972; Richards and Thompson, 1952;

Yentsch and Menzel, 1963). However, very little has been done toward the development of a qualitative method of analysis. Yentsch and Yentsch (1979) proposed the use of a ratio of the fluorescence excited at 530 nm to that excited at 450 nm as a qualitatitive distinction between the green algae and the diatoms and dinoflagellates. However, this technique is less than adequate largely due to excitation variability between species within a class. Slight uncorrected background fluctuations also can cause large errors in the calculated ratios. It would seem to be a better approach to compare all the relevant excitation-emission wavelength pairs, which would not be subject to such variability. Recently, a new method was developed (Rossi and Warner, 1985) that uses correlation analysis for spectral matching of data in the frequency domain. This technique has proven to be an efficient method for the identification of single component fluorescent samples by comparison to a library of known spectra. A preliminary study (Warner et al., 1984) of the application of this method to the pattern recognition of marine phytoplankton has already been described.

Therefore, fluorescence has been demonstrated to provide both the sensitivity necessary for the in situ detection of pigments in natural water samples (Lorenzen, 1966; Flemer, 1969; Kiefer, 1973) and the selectivity needed for phytoplankton population characterization (Yentsch and Yentsch, 1979; Mumola et al., 1975). It also possesses the capabilities of continuous monitoring (Lorenzen, 1966) and/or remote sensing (Mumola et al., 1975). However, until recently, the advantages of fluorescence detection could not be fully exploited due to instrumental limitations. Commercially available fluorometers that use scanning

monochromators do not have the data acquisition speed necessary for continuous monitoring and are usually plaqued by multiple order light scattering that constitutes a significant interferent at low analyte concentrations. However, a new multichannel fluorometer has been introduced (Oldham et al., 1984; Warner et al., 1984) that is similar to the one envisioned by Yentsch and Yentsch (1979) for the continuous monitoring of oceanic phytoplankton. This fluorometer uses an intensified linear photodiode array and is capable of rapid acquisition of EEMs at detection limits of parts-per-trillion for chlorophyll a. Therefore, the instrumentation and data handling methods are now available for the investigation of natural phytoplankton populations, both qualitatively and quantitatively.

This paper presents the use of EEMs as spectral fingerprints for marine phytoplankton populations. Two-dimensional Fourier-transform-based pattern recognition is described along with its inherent strengths and weaknesses for the analysis of natural populations. The EEMs of unknown algae are compared to a library of standard EEMs representing 23 algal species and 6 classes. The EEMs acquired under different physiological conditions are used in determining pattern recognition reliability. The potential for fingerprinting mixed populations and oceanographic regions is also discussed.

Theory

In order to better understand the method of pattern recognition that is demonstrated in this paper, some basic aspects of Fourier data analysis must be described. The general equation for the two-dimensional, forward

Fourier transformation of a given function g(x,y) has been described in the literature (Rossi and Warner, 1984) and is given by Eq. 1.

$$G(u,v) = [F\ g(x,y)] = \int_{-\infty}^{+\infty}\int g(x,y)\ \exp[(-2\pi i(xu + yv)]\ dx\ dy \quad (1)$$

This is called the forward transform because it inverts the physical dimensions of the periodic data set from time to frequency. The inverse transform is described by Eq. 2 and, as the name implies, it simply reverses the direction of the forward transform.

$$g(x,y) = F^{-1}[G(u,v)] = \int_{-\infty}^{+\infty}\int G(u,v)\ \exp[(2\pi i(xu + yv)]\ du\ dv \quad (2)$$

These operations are complex in that they both use and generate complex numbers with real and imaginary components.

The aspect of Fourier analysis that allows spectral matching is called correlation. Correlation can be calculated either in the time or frequency domain but due to computation efficiency the latter method is generally selected. If, for example, s(x,y) represents a standard EEM from a spectral library and u(x,y) represents an unknown EEM, then the time domain correlation function, c(x,y), can be calculated by multiplying the transformed standard spectrum, S(u,v), by the complex conjugate of the transformed unknown spectrum, $U^*(u,v)$, followed by the inverse transformation of the product. The correlation theorem of Fourier transforms is described mathematically by

$$F[c(x,y)] = C(u,v) = S(u,v) \cdot U^*(u,v) \quad (3)$$

where the superscript asterisk indicates the complex conjugate of the function. Two distinct types of correlation are possible. There is the autocorrelation case in which the two functions are equal, i.e., $s(x,y) = u(x,y)$. This, of course, would constitute an exact spectral match. Cross-correlation occurs when the two functions are not equal, i.e. $s(x,y) \neq u(x,y)$. The goal in pattern recognition is to determine which case is present, autocorrelation or cross-correlation. Therefore, some specific mathematical indicators that are capable of indexing samples by spectral similarity must be utilized. Three such indicators used in this paper are 1) the sum, I, of the absolute values of the imaginary coefficients in the Fourier transformed correlation function 2) the sum, R, of the negative real coefficients in the Fourier transformed correlation function and 3) the intervector distance, D, between the abbreviated frequency domain spectra. The intervector distance is defined by

$$D = \left\{ \sum_{u=10}^{10} \sum_{v=10}^{10} [R(u,v) - R'(u,v)]^2 + [I(u,v) - I'(u,v)]^2 \right\}^{1/2} \qquad (4)$$

where R(u,v) and I(u,v) are the real and imaginary coefficients of the frequency domain spectra. The unknown spectral coefficients are marked with a prime. By all three criteria, the autocorrelation or ideal spectral match is indicated by a value of 0. Therefore, spectral matches can be assigned by the absolute magnitudes of the 3 indicators. Generally, one indicator is chosen to rank the spectral match, but in cases where there is ambiguity among the indicators the other two are used to judge spectral similarity.

Methods

The algae used in this study were selected to provide a representative sampling of the various classes of marine algae. A listing of the algal species investigated is provided in Table I. Listings of the respective algal classifications, culture media, and sources are also given. Those species indicated by an asterisk were grown in our laboratory with a 12 hr./12 hr. light/dark cycle except for the blue-greens which were kept in low continuous light. Light of 500 lux was supplied by cool white fluorescent lamps. Provasoli's ASP 6 artificial seawater medium was adjusted to a pH of 7.6 with concentrated HCl. The temperature fluctuated between 16 °C and 25° C. Growth of the cultures used in the physiological variability study was monitored daily with an absorbance spectrophotometer at 450 nm and the growth curves were graphically recorded as in Fig. 1. Samples to be used for spectral analysis were withdrawn from the culture tubes during the appropriate growth phase. In order to reduce possible contamination, replicate tranfers were made so that a single culture tube was only sampled once.

The EEMs used for pattern recognition were acquired by a portable multichannel fluorometer (Oldham et al., 1984; Warner et al., 1984). This instrument uses a 512 element intensified linear photodiode array that is water cooled for the reduction of dark current noise. Excitation is provided by an AC stabilized (Oldham et al., 1985) 150 watt DC xenon arc lamp. Individual wavelengths are selected by a stepping motor driven circular variable filter wheel. The phytoplankton sample is recorded in vivo by pipetting the culture into a standard 1 cm quartz fluorescence

cuvet. Emission spectra are obtained at various excitation wavelengths and then organized into an EEM format by the controlling Apple II+ microcomputer (Apple Computer, Cupertino, California). The EEMs are stored on a hard disk and later transferred to a Hewlett-Packard 9845B minicomputer (Hewlett-Packard, Palo Alto, California) for the pattern recognition analysis.

Results and Discussion

Representative EEMs of the different algal classes examined in this study are shown in Fig. 2. The three pigment systems (chlorophylls, carotenoids, and phycobilins) previously described are all present in various proportions in Fig. 2. Table II describes the general pigment composition of these algae. The distribution of pigments provides for sharp distinction between the greens, blue-greens, and those (diatoms, dinoflagellates and golden-browns) that contain a carotenoid complex. This distinction is immediately obvious upon examination of the EEMs in Fig. 2. It is also possible to differentiate between certain species within a class by visually examining their EEMs as in Fig. 3. In this case, the spectra from two blue-green algae are shown. Figure 3A indicates phycocyanin as the major fluorophore and 3B indicates phycoerythrin. Except for the phycobilins in the blue-greens, the secondary pigments transfer their excitation energy to chlorophyll a, which releases a portion of this energy by fluorescence. The carotenoids do not exhibit fluorescence *in vivo* or in solution. However, due to the transfer of excitation energy from the carotenoids to chlorophyll a, the excitation spectrum of the carotenoids is detectable via the emission of chlorophyll

a. Figure 4 illustrates the decoupling of the energy transfer after the cellular pigments are extracted into a suitable solvent. The extracted EEM (Fig. 4B) has obviously lost the carotenoid peak but a small chlorophyll c peak has been introduced. Thus, the information content of the in vivo and the extracted EEMs can be complementary to each other.

Even though it is possible in some cases to visually distinguish between the in vivo spectra of different species, many of those examined are too similar to each other in their pigment composition for this to always be possible. Therefore, the correlation method previously described is used for the pattern recognition of in vivo EEMs. This method provides an objective judgment, based on all excitation-emission wavelength pairs, concerning spectral similarity. In the preliminary study (Warner et al., 1984), replicate EEMs from 9 different species were acquired. One set of EEMs was arbitrarily used as the standard library while the other set was used as unknowns. In each case, the correct standard was selected by the pattern recognition software as the most similar EEM. However, since these were replicate spectra acquired on the same day, a study of the physiological condition of the cell population is undertaken here.

Seven species were cultured in our laboratory under the conditions previously described. A spectrum of each species was acquired during the logarithmic, stationary, and senescent phases of growth. The various growth phases allowed samples to be examined spectrally under different physiological conditions. The acquired EEMs were compared to the same standard library as in the previous study. The results of the pattern recognition are tabulated in Table III and displayed graphically in Fig. 5.

The degree of spectral similarity of each standard in the spectral library with the unknown spectrum is ranked numerically with the most similar standard being hit #1. The hit rankings listed in Table III correspond to the rank assigned to the correct standard by the pattern recognition program. Good agreement is shown despite the fact that the samples were acquired weeks apart and under different physiological conditions. This indicates that although fluorescence intensity may vary a great deal with cell physiology, the spectral distribution remains generally consistent. In the cases where the correct spectral match appears lower than the first hit, it should be noted that the standards ranked higher (previous hits) are generally very similar in pigment composition to the correct standard. With better selection of standard spectra, a further improvement in recognition accuracy could possibly be achieved.

Because it is difficult to generalize with a standard library of only 9 species, the library was supplemented with 23 additional species obtained from the culture collection at Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine. In a new study using the expanded spectral library, replicate spectra obtained for each culture were acquired on different days. One set of EEMs was again used as the standard library and the other set as unknowns. The results of the spectral matching are presented in Fig. 6. As in the other studies, in those cases where the correct match did not appear first on the list, the standards that preceded it were spectrally similar. If gross characterization is all that is desired, it appears that a valid approach to interpreting the data would be to examine the first three hits. If at least two of the three hits are

from the same class of algae, then it is reasonable to place the unknown sample in that class as well. When this criterion is administered to the previous data, better than an 80% accuracy is calculated.

Since marine phytoplankton rarely exist in homogeneous communities, the projection of such a pattern recognition technique to natural oceanographic samples mandates the examination of mixtures. For this purpose, two standard EEMs of unialgal cultures (Chlorella vulgaris and Amphidinium carterae) were synthetically added to each other in varying proportions from 0% C. vulgaris fluorescence to 100% C. vulgaris fluorescence in 10% increments. The proportions were calculated based on the maximum fluorescence intensity. The series of mixtures was then correlated against the original 9 standard library in which the C. vulgaris and A. Carterae EEMs were represented. Table IV presents the results of the pattern recognition of these mixtures. It is encouraging to note that the matching accuracy at the two ends of the mixture series is very good. The general trend is that as the contribution from the second component increases, the numbers approach each other until they cross at approximately the point of equifluorescence (40-60%). This trend continues with the numbers separating as the second component approaches 100%. Therefore, the points of maximum ambiguity in the pattern recognition occur in the region where the two components contribute similar spectral intensities to the mixed spectrum.

Conclusions

The results of these studies indicate that the frequency domain correlation of EEMs is an accurate and reliable method of algal recognition. The multichannel fluorometer used has been shown to provide the multiple wavelength selectivity of commercially available fluorometers without sacrificing data acquisition speed or sensitivity. Thus, the potential for characterizing natural phytoplankton populations by fluorescence spectroscopy now exists. The powerful combination of rapid scanning instrumentation and pattern recognition methods allows for the total characterization (qualitative and quantitative) of natural waters. Not only is there the potential for the recognition of algal types in natural samples by fluorescence, but there is also the possibility of fingerprinting local regions of interest. Thus, the oceanographer now has the means available to detect dynamic relationships of regions and populations by spectral changes.

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TABLE I.

List of algal species investigated.

Class	Species	Source[†]	Media
Chlorophyceae	Chlorella vulgaris *	1	ASP 6
	Dunaliela sp. *	2	ASP 6
	Chlorella capsulata	4	F/2
	Dunaliela tertiolecta	4	F/2
	Chlamydomonas sp.	4	F/2
	Chlorosarcinopsis halophilia	4	F/2
Prasinophyceae	Tetraselmis sp.*	1	ASP 6
Bacillariophyceae	Bellerochea horologicalis *	1	ASP 6
	Skeletonema costatum *	2	ASP 6
	Thalassiosira weissflogii	4	F/2
	Phaeodactylum tricornutum	4	F/2
	Chaetoceros gracile	4	F/2
	Thalassiosira pseudonann	4	F/2
	Skeletonema costatum	4	F/2
Dinophyceae	Amphidinium carterae *	3	ASP 6
	Gymnodinium simplex	4	F/2
	Scrippsiella trochoidea	4	F/2
	Prorocentrum minimum	4	F/2
	Prorocentrum micans	4	F/2
	Heterocapsa triquetra	4	F/2

TABLE I. Continued

Class	Species	Source†	Media
Cyanophyceae	Spirulina major*	3	ASP 6
	Synechococcus sp.	4	F/2
	Synechococcus sp.	4	F/2
	Synechococcus bacillaris	4	F/2
	Oscillatoria woronichini	4	F/2
	Phormidinium perscienium	4	F/2
Prymnesiophyceae	Prymnesium parvum*	1	ASP 6
	Coccolithophora sp.	2	soil-seawater
	Emilianin huxleyi	4	F/2
	Pavlova lutheri	4	F/2
	Isochrysis galbana	4	F/2
	Hymenomonas carterae	4	F/2
Chloromonadophyceae	Chattonella luteus	4	F/2

†1) Dr. Gabe Vargo, Department of Biology, University of South Florida

2) Dr. G. Frexell, Department of Oceanography, Texas A&M University

3) Carolina Biological Supply Company, Burlington, North Carolina

4) Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine

TABLE II.

Pigment composition by algal class.

Class	Common Name	Chlorophylls a	b	c	Carotenoids	Phycobilins
Cyanophyceae	Blue-Green	+				+
Dinophyceae	Dinoflagellate	+		+	+	
Prymnesiophyceae	Golden-Brown	+		+	+	
Bacillariophyceae	Diatom	+		+	+	
Chlorophyceae	Green	+	+			

TABLE III.

Effects of cell physiology
on spectral matching.

		HIT #		
Type	Culture	Log Phase	Stat. Phase	Senescent Phase
Green	C. vulgaris (C)	1	2 (D)	2 (D)
Green	D. sp. (D)	1	1	1
Dinoflagellate	A. carterae (A)	2 (SK)	1	2 (B)
Golden-Brown	P. parvum (P)	2 (A)	2 (A)	2 (A)
Diatom	S. costatum (SK)	1	3 (A,B)	4 (CL,B,A)
Green (?)	T. sp. (T)	3 (C,D)	1	1
Blue-Green	S. major (SP)	1	1	1
Diatom	B. horologicalis (B)	-	-	-
Golden-Brown	C. sp. (CL)	-	-	-

Note: ( ) = Previous Hits

TABLE IV.

Pattern recognition results of a synthetic binary mixture.

% C. vulgaris	HIT #	
	A. carterae	C. vulgaris
0	1	4
10	1	4
20	1	3
30	2	3
40	2	2
50	3	1
60	4	1
70	4	1
80	4	1
90	4	1
100	4	1

Figure Captions

1. Growth curve for *Skeletonema costatum*. λ_{abs} = 450 nm.
2. Typical EEMs representing 5 algal classes.
   A) Cyanophyceae
   B) Chlorophyceae
   C) Prymnesiophyceae
   D) Dinophyceae
   E) Bacillariophyceae.
3. *In vivo* EEMs representing the two pigment systems present in blue-green algae.
   A) *Synechococcus bacillaris*
   B) *Oscillatoria woronchini*.
4. Contour plots of *Amphidinium carterae* pigment fluorescence.
   A) *In vivo*
   B) Extracted into 90% acetone/water.
5. Effects of cell physiology on spectral matching.
   A) Logarithmic phase
   B) Stationary phase
   C) Senescent phase.
6. Results of spectral matching on extended data base.

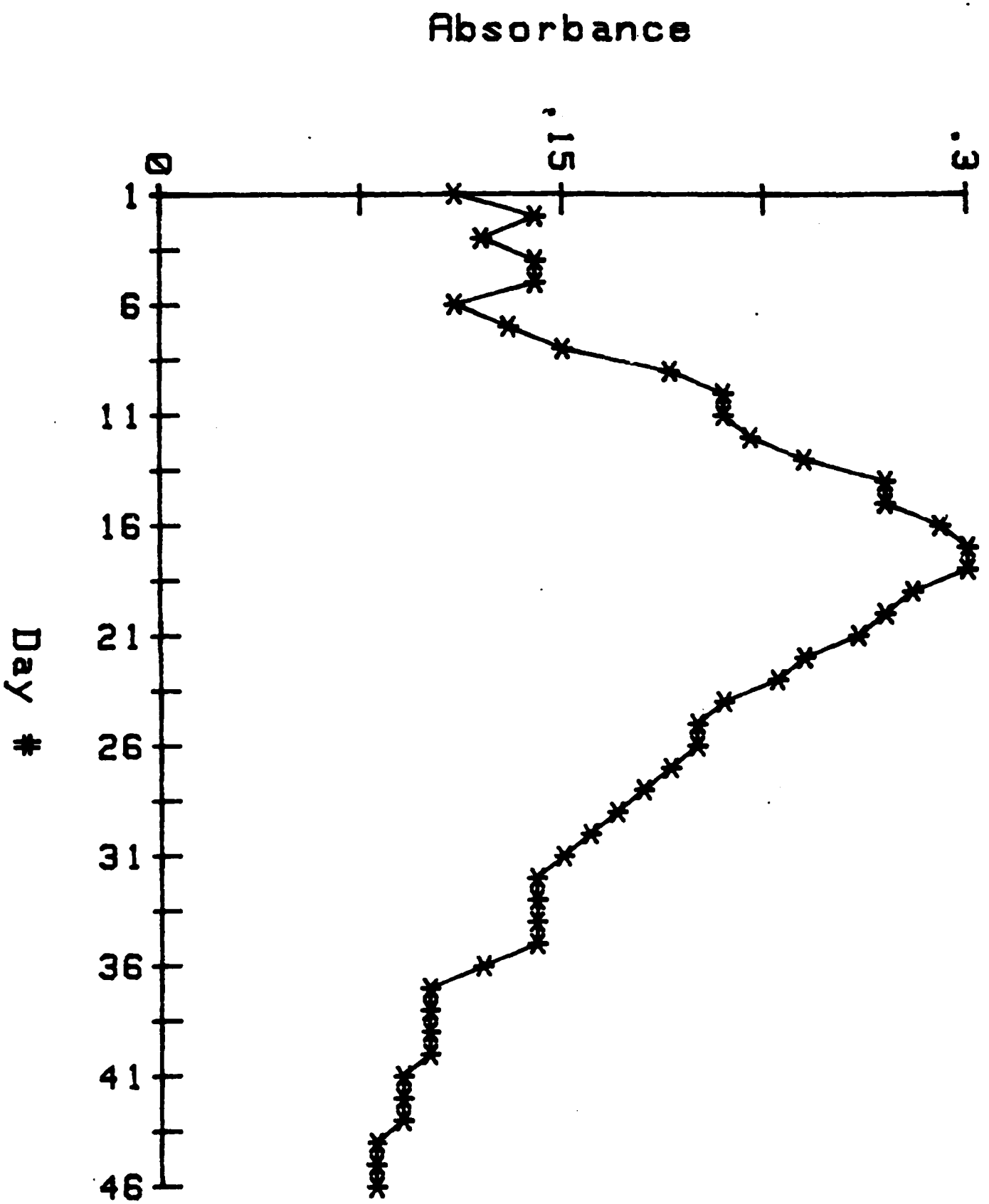

Figure 1

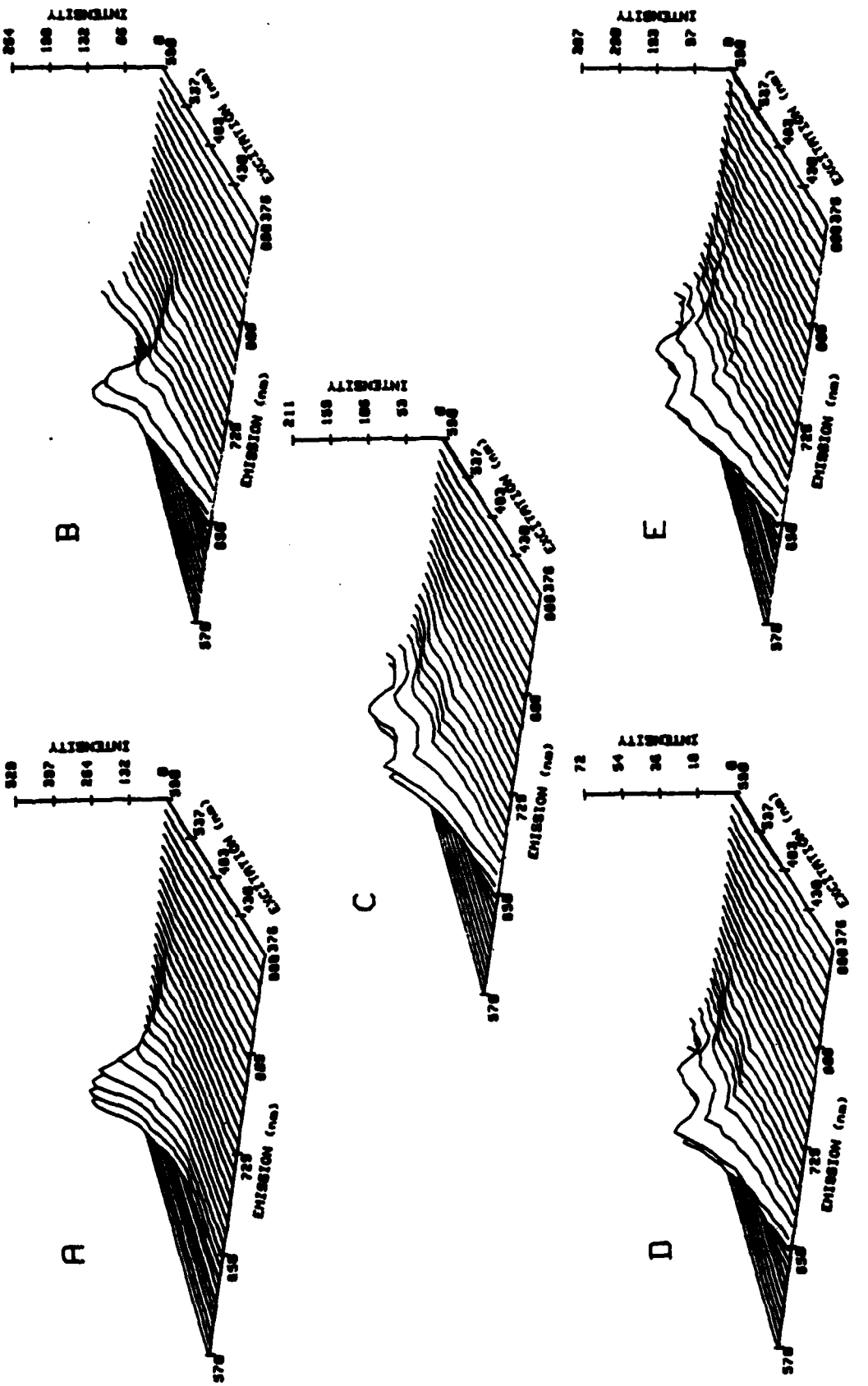

Figure 2

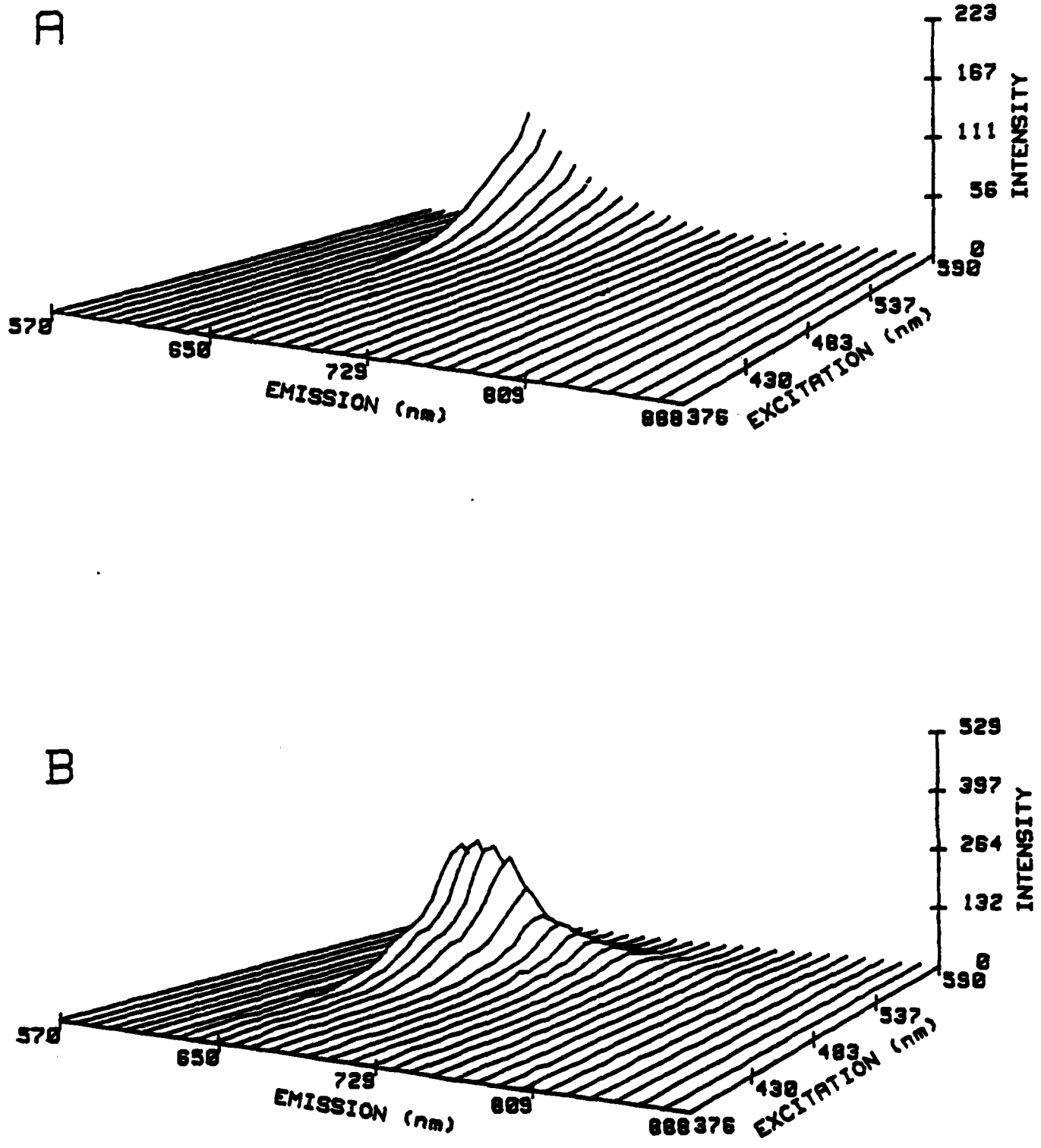
A
223
167
111
56
0
INTENSITY
590
537
483
430
376
EXCITATION (nm)
570
650
729
809
EMISSION (nm)
B
529
397
264
132
0
INTENSITY
590
537
483
430
376
EXCITATION (nm)
570
650
729
809
EMISSION (nm)

Figure 3

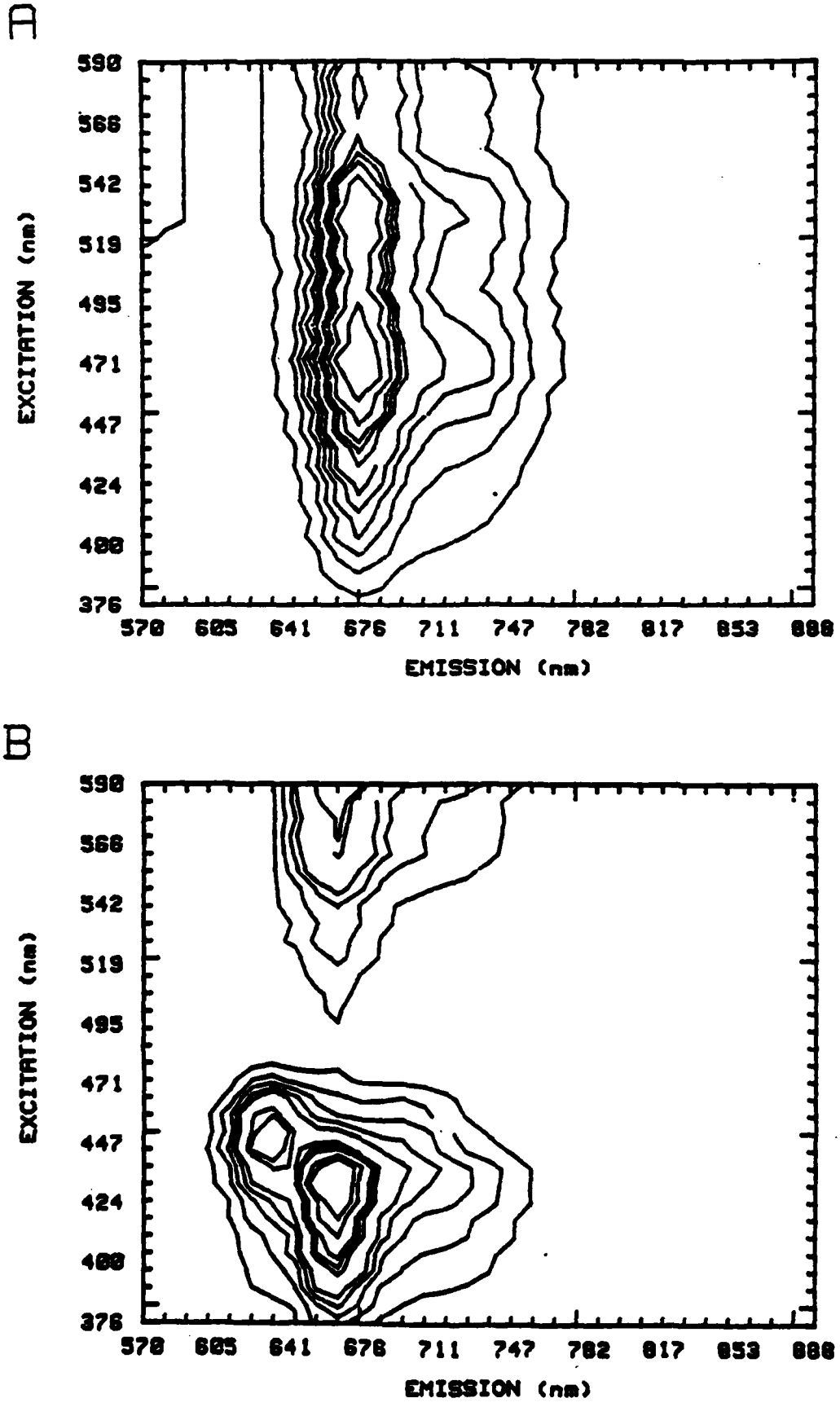
A
590
566
542
519
495
471
447
424
400
376
EXCITATION (nm)
570
605
641
676
711
747
782
817
853
888
EMISSION (nm)
B
590
566
542
519
495
471
447
424
400
376
EXCITATION (nm)
570
605
641
676
711
747
782
817
853
888
EMISSION (nm)

Figure 4

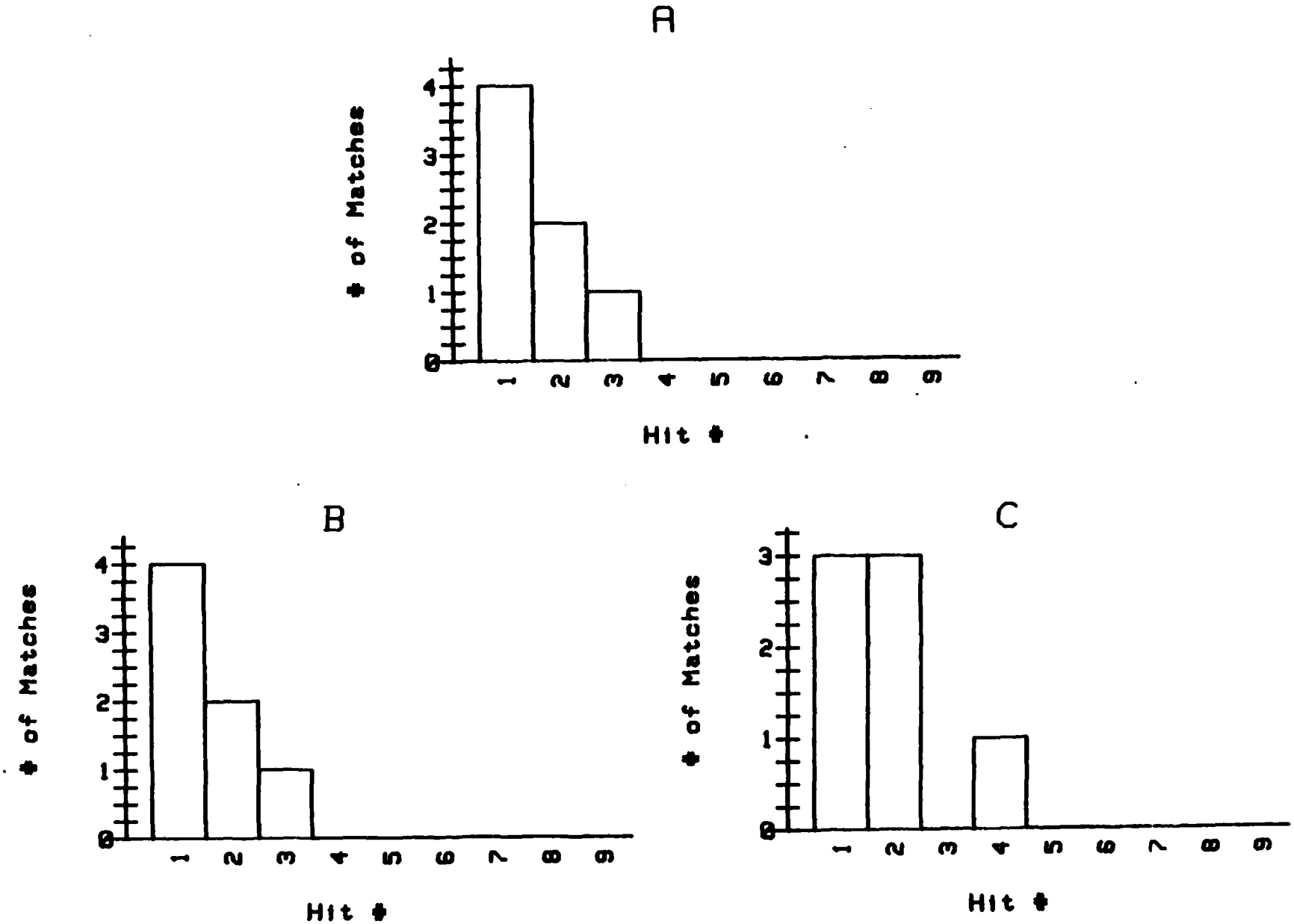
A
# of Matches
0
1
2
3
4
1 2 3 4 5 6 7 8 9
Hit #
B
# of Matches
0
1
2
3
4
1 2 3 4 5 6 7 8 9
Hit #
C
# of Matches
0
1
2
3
1 2 3 4 5 6 7 8 9
Hit #

Figure 5

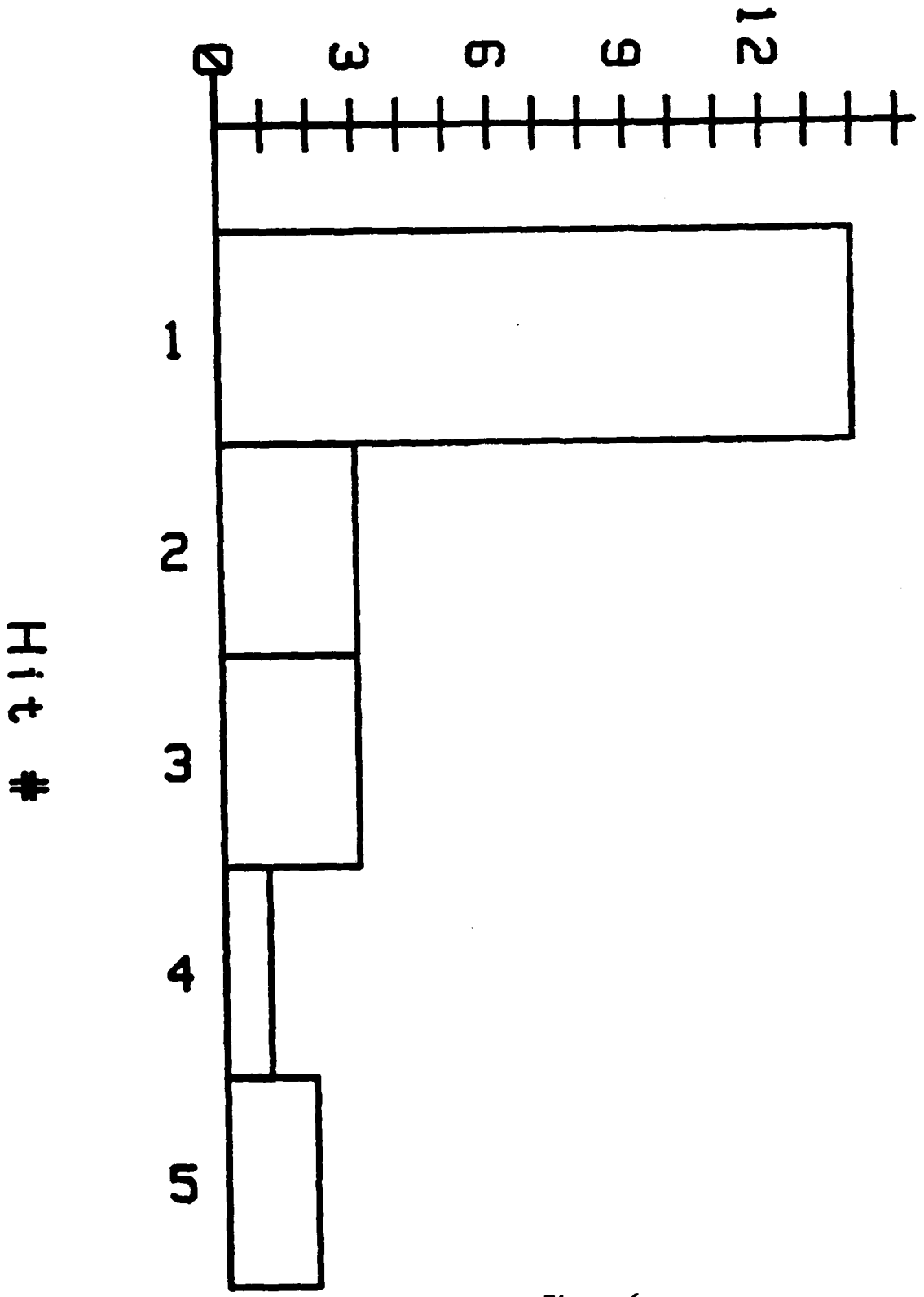

Figure 6

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Spectral "Fingerprinting" of Phytoplankton Populations by Two-Dimensional Fluorescence and Fourier-Transform-Based Pattern Recognition

by

Philip B. Oldham, E.J. Zillioux,

Isiah M. Warner

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